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Fibroblast growth factors (FGF) have been shown by gain and loss of function experiments to play critical roles in development and tumorigenesis. FGFs are expressed during all stages of mouse mammary gland development and in humans dysregulated expression has been correlated with poor prognosis breast cancer. The focus of this research is to study how FGFs function during mammary gland development and what role they play in the etiology of breast cancer. We have developed and characterized a system of FGF-independent inducible activation of fibroblast growth factor receptors (FGFR). The intracellular kinase domains of all four FGFRs have been cloned together with a FVBP domain that can induce dimerization in the presence of the lipid soluble drug AP20187. NIH3T3 fibroblasts stably transfected with the FGFR-FVBP constructs can be AP20187-dependently rescued from serum deprivation induced apoptosis as measured by MTS and caspase-3 activation assays. Dimerization of FGFR-FVBPs leads to phosphorylation of themselves and downstream signaling factors including ERK1/2 and AKT. Anti-apoptotic Bcl family members Bcl2 and Bcl-xl mRNA are upregulated by FGFR-FVBP activation. From these data we propose that FGFRs can block apoptosis in NIH3T3 cells through post-translational and transcriptional pathways. Transgenic mice that express these constructs in the mammary gland are currently being characterized

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FOREWORD

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BV X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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Introduction

Fibroblast growth factors (FGF) are potent growth factors involved in many developmental events during embryogenesis. FGFs play a role primary mesoderm induction, limb bud outgrowth and patterning, and lung alveogenesis. During these developmental events the expression of FGFs are restricted and regulated. The aberrant expression of FGFs outside these regulated confines has been correlated with tumorigenesis. The oncogenic roles of FGFs in mammary tumorigenesis were initially identified by the finding that mouse mammary tumor virus (MMTV) insertion can cooperatively activate Wnt-1 and FGF3 in tumors of MMTV infected mice. Two other FGF family members have also been identified that cooperate with Wnt-1 to induce mammary gland tumorigenesis: FGF4 and FGF8. We are interested in elucidating the function of aberrant expression of FGFs in mammary tumorigenesis. Overexpression of FGFs has been shown to induce mammary tumorigenesis in mouse models and has been postulated to play a role in human breast cancer. Dysregulation of FGFs and FGFRs has been reported in human prostate and breast cancers and is associated with poor prognosis. Understanding the mechanism of FGFs and FGF receptors (FGFR) in breast cancer may provide insight for new therapeutics derived to block FGF/receptor interactions or receptor tyrosine kinase activity. The goal of this project is to understand the mechanism of mammary oncogenic FGF8 and FGF receptors in tumorigenesis. To attain this goal we have developed a system for FGF-independent inducible activation of the FGFR to study the FGF signaling pathway *in vitro* and *in vivo.*

Body

Specific Aims

We have modified our original specific aims as explained in our 1998-99 progress report to address new developments in the field and to broaden our studies on FGF function in the mammary gland. Our current specific aims are:

1) To determine the spatial and temporal expression of Fgf-8 during normal mammary gland development and in tumors.

2) To characterize the cooperative potential of Fgf-8 and beta-catenin in mammary tumorigenesis.
3) Identify genes that are regulated by FGF signaling.
4) Determine the oncogenic potential of the FGFRs in mammary gland tumorigenesis.

Progress toward these aims:

Specific Aim 1: To determine the spatial and temporal expression of Fgf-8 during normal mammary gland development and in tumors.

We have previously described our analysis of FGF8 expression in the developing mammary gland and tumors (progress report 1998-99). Briefly, our data show that multiple isoforms of FGF8 are expressed during pregnancy and lactation in the normal mammary gland by RT-PCR analysis that can resolve all FGF8 isoforms. FGF8 is also overexpressed in mammary gland tumors derived from transplanted DIM3 and D2-HAN mammary epithelial cell lines. We have determined that FGF8 mRNA expression is restricted to the mammary gland epithelium using cell fractionation and RT-PCR analysis. The temporal and spatial expression pattern of FGF8 was described in our last progress report and no new progress has been made toward this aim in the past year. These data will be combined with data from specific aim 2 for publication.

Specific Aim 2: To characterize the cooperative potential of Fgf-8 and beta-catenin in mammary tumorigenesis.

FGF and Wnt growth factors have been shown to have cooperative effects at inducing mammary gland tumorigenesis in mouse models. To study the FGF/Wnt cooperatively we are optimizing a system for introducing genes into the mammary gland by a retroviral gene transfer method. In this procedure we isolate mammary gland primary cultures, infect them over the course of 4 days with retroviral containing media and transplant the infected cells into syngeneic host mice. The mammary gland outgrowths are then analyzed for morphological and molecular differences. During the past year we have significantly optimized our retroviral transfer method by increasing our transduction efficiency from 15% to 85-90% of mammary epithelial HC11 cells. In the modified procedure we utilize polybrene with a centrifugation step to dramatically increase our infection rates. We are also elucidating the transduction efficiency of several different psuedotyped retroviruses containing different envelope proteins including ecotropic,

amphotropic, 10A1, and VSVg. Each psuedotyped retroviruses has a different specificity for cell surface receptors allowing the transduction of a variety of cell types. We have optimized these constructs in terms of their ability to infect HC11 cells and we are now characterizing them for efficient transduction of mammary epithelial primary cultures. In these experiments we are using a retrovirus containing a Lac-Z gene as a reporter for determining transduction rates in culture and transplanted outgrowths. Once we have optimized the protocol using the Lac-Z retrovirus we will infect and transplant primary cultures using FGF8 and modified beta-catenin retroviruses. The modified beta-catenin contains N-terminal GSK-3 beta phosphorylation site mutations that stabilize the protein. The outgrowth s will be characterized for morphological and molecular changes in ductal structure, apoptosis and proliferation. Optimization of the retrovirus infection protocol should be completed in the first half of the 2000-01 year followed by the FGF8/beta-catenin experiments. We hope to publish the optimization procedure as a method paper and combine the data from the FGF8/beta-catenin transplant experiments with specific aim 1 for publication.

Specific Aim 3: Identify genes that are regulated by FGF signaling.

All four FGFR intracellular kinase domains have been cloned with FvBP dimerization domains and are being used to elucidate signaling characteristics in NIH3T3 fibroblasts and HC11 mammary epithelial cells. The rationale and experimental design were explained in the previous progress report. FGFR1-3 have been most extensively studied for there ability to block apoptosis induced by serum deprivation in NIH3T3 cells. We have utilized two assays to study apoptosis, an MTS assay for determining cell number and Caspase-3 activation assay. All three receptors block apoptosis in serum starved stably transfected NIH3T3 cells in an AP20187 dependent manner. AP20187 treatment of these cells results in rapid phosphorylation of the FGFR-FVBP constructs and downstream signaling factors Erk1/2 and AKT. Although FGFR signaling can efficiently block apoptosis and activate the RAS signaling pathway the cells remain quiescent and are arrested in G0/G1 of the cell cycle. Activation of the FGFR-FVBP constructs also results in the upregulation of the anti-apoptotic factors Bcl-2 and Bcl-xl mRNA. From these data we propose that FGFR signaling for FGFRs 1-3 is redundant and can block apoptosis of serum starved NIH3T3 cells through post-translational and transcriptional regulation of survival factors. We are currently characterizing FGFR4-FvBP in NIH3T3 cells to complete the repertoire of FGFR kinases. Once we have completed the

experiments on all four FGFR-FVBP constructs (first half of 2000-01) we will submit the data for publication.

The FGFR-FVBP constructs will be used to determine differences and similarities in signaling between NIH3T3 fibroblasts and HC11 mammary epithelial cells. We will determine if FGF signaling can activate AKT, inhibit Caspase-3 and block apoptosis in HC11 cells similar to that observed in fibroblasts. We will also analyze cell cycle profiles for AP20187 treated HC11 cells to determine if FGF signaling can induce cell proliferation.

Specific Aim 4: Determine the oncogenic potential of the FGFRs in mammary gland tumorigenesis.

To characterize FGF signaling *in vivo* we have developed transgenic mice that express FGFR1-FVBP and FGFR2-FVBP in the mammary gland. The transgene contains the MMTV promoter driving the expression of the FGFR-FVBP genes. Transgenic founder lines were determined by PCR and Southern blot analyses. We have at least one FGFR1-FVBP and two FGFR2-FVBP lines that express the constructs in the mammary gland as determined by Western blot analyses. These lines are currently being expanded for use in experiments. The transgenic mice will be given courses of intraperitoneal injections of AP20187 during critical stages of mammary gland development to elucidate the developmental effects of FGFR signaling. Long term studies on the oncogenic effect of FGFR signaling will utilize slow release pellets implanted in the mammary gland. These studies will be ongoing for the entire 2000-01 year.

Key Research Accomplishments

1) Developed and characterized a system for FGF-independent inducible activation of FGFRs.

2) Determined that FGFR signaling has redundant capabilities at blocking serum-deprivation induced apoptosis in NIH3T3 fibroblasts.

-FGFR1-3 signaling can inhibit apoptosis and activate Erk1/2, AKT and inhibit caspase-3.

-FGFR activation can upregulate the mRNA for the anti-apoptotic genes Bcl-2 and Bcl-xl.

-FGFR1-3 cannot induce proliferation in serum starved fibroblasts.

3) Developed transgenic mice that express the FGFR1- and 2-FVBP constructs to use as an *in vivo* model for FGFR signaling in the mammary gland.

4) Optimized retroviral transduction of HC11 mammary epithelial cells.

Reportable Outcomes

Data from this project was presented at two institutional and one international symposia.

1) Keystone Symposia, Signaling 2000. Keystone, Colorado. January 22-28, 2000
2) Graduate Student Day Symposium, Baylor College of Medicine, March 19, 2000
3) Cell and Molecular Biology Research Conference and Retreat, Baylor College of Medicine, March 31-April 1, 2000

Conclusions

We have made progress in the past year developing and characterizing a novel method to study FGFR signaling. We are using this system in *in vitro* and *in vivo* experiments to elucidate the oncogenic potential and developmental mechanisms of FGFR signaling in the mammary gland. We are anticipating compiling much of the collected data from the past two years into manuscripts to be submitted for publication in 2000-01.

Inducible ligand-independent activation of fibroblast growth factor receptors as a method to study mammary gland tumorigenesis.

Welm, B., Freeman, K*., Wang, J., Spencer, D*., Rosen, J., Departments of Molecular and Cellular Biology and Microbiology and Immunology*, Baylor College of Medicine, Houston Texas, 77030

We have developed a system to functionally study fibroblast growth factor receptors (FGFR) in an inducible ligand-independent system. FGFRs consist of a family of four highly homologous genes that are potent regulators of proliferation, mitogenesis, angiogenesis and apoptosis. We have constructed and characterized chimeric proteins that contain only the intracellular domains of all four FGFR genes linked to a modified FKBP gene. The FGFR-FKBP constructs are myristlated for membrane targeting and tagged with the hemagglutinin epitope. These chimeric proteins can be induced to homodimerize in the presence of the lipid soluble drug AP20187. AP20187 treatment of NIH3T3 cells stably transfected with the FGFR-FKBP constructs results in FGFR-FKBP tyrosine phosphorylation. We have also observed inducible phosphorylation of downstream targets of FGFR signaling including MAPK, AKT, and GSK3β. FGFR-FKBP cells treated with AP20187 can block apoptosis induced by serum deprivation as measured by caspase-3 inactivation and tunel assays. In addition we have observed inducible mRNA up-regulation of the anti-apoptotic factors BCL-2 and BCL-xl. To study FGFR function during mammary gland development and tumorigenesis these FGFR-FKBP chimeric constructs, under the control of the MMTV promoter, have been used to develop transgenic mice. (B.W. is supported by DOD fellowship DAMD17-98-1-8283)

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Signaling 2000 (B1)
Poster Session #1

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